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REGULATION OF CYTOKINE SYNTHESIS AND RELEASE

Abstract:

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Methods of treatment and prevention of diseases associated with release of neutrophil elastase and IL-8 by administration of TFPI, and analogs of TFPI are disclosed. Methods of determining efficacy of treatment with TFPI, patient's responsiveness to treatment with TFPI and the ultimate determination of patient prognosis are also disclosed. Data supplied from the esp@cenet database - Worldwide

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(54) Title: REGULATION OF CYTOKINE SYNTHESIS AND RELEASE

(57) Abstract

Methods of treatment and prevention of diseases associated with release of neutrophil elastase and IL-8 by administration of TFPI, and analogs of TFPI are disclosed. Methods of determining efficacy of treatment with TFPI, patient's responsiveness to treatment with TFPI and the ultimate determination of patient prognosis are also disclosed.

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REGULATON OF CYTOKINE SYNTHESIS AND RELEASE

Technical Field

The invention relates to the use of Tissue Factor Pathway Inhibitor (TFPI) to inhibit the synthesis and release of neutrophil elastase, and IL-8 and inhibit the activity of plasmin.

Background of the Invention

Tissue Factor Pathway Inhibitor (TFPI) inhibits the coagulation cascade in at least two ways: preventing formation of factor VIIa/tissue factor complex and by binding to the active site of factor Xa. The primary sequence of TFPI, deduced from cDNA sequence, indicates that the protein contains three Kunitz-type enzyme inhibitor domains. The first of these domains is required for the inhibition of the factor VIIa/tissue factor complex. The second Kunitz-type domain is needed for the inhibition of factor Xa. The function of the third Kunitz-type domain is unknown. TFPI has no known enzymatic activity and is thought to inhibit its protease targets in a stoichiometric manner; namely, binding of one TFPI Kunitz-type domain to the active site of one protease molecule. The carboxy-terminal end of TFPI is believed to have a role in cell surface localization via heparin binding and by interaction with phospholipid. TFPI is also known as Lipoprotein Associated Coagulation Inhibitor (LACI), Tissue Factor Inhibitor (TFI), and Extrinsic Pathway Inhibitor (EPI).

Mature TFPI is 276 amino acids in length with a negatively charged amino terminal end and a positively charged carboxy-terminal end. TFPI contains 18 cysteine residues and forms 9 disulphide bridges when correctly folded. The primary sequence also contains three Asn-X-Ser/Thr N-linked glycosylation consensus sites, the asparagine

residues located at positions 145, 195 and 256. The carbohydrate component of mature TFPI is approximately 30% of the mass of the protein. However, data from proteolytic mapping and mass spectral data imply that the carbohydrate moieties are heterogeneous. TFPI is also found to be phosphorylated at the serine residue in position 2 of the protein to varying degrees. The phosphorylation does not appear to affect TFPI function.

TFPI has been isolated from human plasma and from human tissue culture cells including HepG2, Chang liver and SK hepatoma cells. Recombinant TFPI has been expressed in mouse C127 cells, baby hamster kidney cells, Chinese hamster ovary cells and human SK hepatoma cells. Recombinant TFPI from the mouse C127 cells has been shown in animal models to inhibit tissue-factor induced coagulation.

A non-glycosylated form of recombinant TFPI has been produced and isolated from *Escherichia coli* (*E. coli*) cells as disclosed in U.S. Pat. No. 5,212,091. This form of TFPI has been shown to be active in the inhibition of bovine factor Xa and in the inhibition of human tissue factor-induced coagulation in plasma. Methods have also been disclosed for purification of TFPI from yeast cell culture medium, such as in Petersen *et al*, J.Biol.Chem. 18:13344-13351 (1993).

Recently, another protein with a high degree of structural identity to TFPI has been identified. Sprecher *et al*, Proc. Nat. Acad. Sci., USA 91:3353-3357 (1994). The predicted secondary structure of this protein, called TFPI-2, is virtually identical to TFPI with 3 Kunitz-type domains, 9 cysteine-cysteine linkages, an acidic amino terminus and a basic carboxy-terminal tail. The three Kunitz-type domains of TFPI-2 exhibit 43%, 35% and 53% primary sequence identity with TFPI Kunitz-type domains 1, 2, and 3, respectively. Recombinant TFPI-2 strongly inhibits the amidolytic activity of factor

VIIa/tissue factor. By contrast, TFPI-2 is a weak inhibitor of factor Xa amidolytic activity.

TFPI has been shown to prevent mortality in a lethal *Escherichia coli* (*E. coli*) septic shock baboon model. Creasey *et al*, J. Clin. Invest. 91:2850-2860 (1993). Administration of TFPI at 6 mg/kg body weight shortly after infusion of a lethal dose of *E. coli* resulted in survival in all five TFPI-treated animals with significant improvement in quality of life compared with a mean survival time for the five control animals of 39.9 hours. The administration of TFPI also resulted in significant attenuation of the coagulation response, of various measures of cell injury and significant reduction in pathology normally observed in *E. coli* sepsis target organs, including kidneys, adrenal glands, and lungs.

Due to its clot-inhibiting properties, TFPI may also be used to prevent thrombosis during microvascular surgery. For example, U.S. 5,276,015 discloses the use of TFPI in a method for reducing thrombogenicity of microvascular anastomoses wherein TFPI is administered at the site of the microvascular anastomoses contemporaneously with microvascular reconstruction.

Neutrophil elastase release is linked to acute inflammatory diseases including ARDS and multiple organ failure. Idle, *et al*, (1985) Am. Rev. Respire. Ids. 132:1098. Joshua, M., *et al*, (1994) Am. J. Respire. Crate. Care Med. 150:S123. Acute inflammatory reactions, including ARDS, reperfusion injury (including lung reperfusion injury), arthritis, and sepsis are also associated with the production of cytokines such as IL-8. IL-8 is thought to play an important role in the recruitment and activation of PMNs at inflammatory sites.

Currently, there is no single agent which might effectively inhibit both thrombosis due to activation of the extrinsic pathway of coagulation and the release of inflammatory mediators such as neutrophil elastase.

Summary of the Invention

It has now been found that coagulation activation and LPS (the active moiety of bacterial endotoxin) synergize for elastase release and that TFPI inhibits elastase release induced by coagulation activation and by coagulation in the presence of LPS. Further, TFPI has been shown to inhibit plasmin activity at therapeutically relevant doses. Therefore, TFPI has been shown to be relevant and is useful in disease states involving inflammation resulting from elastase release. Accordingly, TFPI may be used to treat such clinical indications as severe acute pancreatitis, emphysema, rheumatoid arthritis, multiple organ failure, cystic fibrosis, Adult Respiratory Distress Syndrome ('ARDS') and sepsis.

It has also been found that coagulation activation/clotting induces IL-8 production in normal human whole blood cultures. Moreover, it has been found that coagulation activation/clotting and LPS together in whole blood cultures synergize for increased IL-8 production. TFPI is able to block the IL-8 production induced under both circumstances. Thus, TFPI may be used to treat such clinical indications as ARDS, reperfusion injury (including lung reperfusion injury), sepsis and arthritis,.

Finally, the observations that TFPI inhibits the activity of plasmin and the synthesis and release of neutrophil elastase and IL-8 allow the use of assays for plasmin activity, and for elastase and IL-8 to be used to determine the patient's response to TFPI.

Brief Description of the Drawings

Figure 1 shows production of neutrophil elastase in undiluted whole blood cultures under the following conditions: control (clot); TFPI (10 μg/ml); LPS (1ng/ml)(clot); TFPI + LPS and heparin (50 u/ml).

Figure 2 shows production of neutrophil elastase in coagulating 1:10 whole blood culture containing various concentrations of hirudin.

Figure 3 shows production of neutrophil elastase in diluted (1:10) whole blood cultures containing various concentrations of TFPI and hirudin or heparin.

Figure 4 shows production of neutrophil elastase in diluted (1:10) whole blood cultures containing 1ng/ml LPS in addition to various concentrations of TFPI and hirudin or heparin.

Figure 5 displays the results of experiments showing that TFPI inhibits plasmin activity.

Figure 6 displays the results of experiments showing that IL-8 levels in whole blood culture are drastically reduced in the presence of TFPI.

Figure 7 shows the synergistic effect of coagulation activation/clotting and LPS in whole blood cultures on IL-8 levels.

Figures 8 and 9 show the results of time course experiments measuring IL-8 levels in whole blood cultures in the absence (Figure 8) and the presence (Figure 9) of LPS.

Figure 10 shows the effect of TFPI on cytokine production in whole blood cultures containing 5 U/ml Hirudin and 1 ng/ml LPS.

Detailed Description of the Invention

A. Definitions

As used herein, "TFPI" refers to mature Tissue Factor Pathway Inhibitor. As noted above, TFPI is also known in the art as Lipoprotein Associated Coagulation Inhibitor (LACI), Extrinsic Pathway Inhibitor (EPI) and Tissue Factor Inhibitor (or TFI). Muteins of TFPI which retain the biological activity of TFPI are encompassed in this definition. Further, TFPI which has been slightly modified for production in bacterial cells is encompassed in the definition as well. For example, a TFPI analog have an alanine residue at the amino-terminal end of the TFPI polypeptide has been produced in *Escherichia coli*. See U.S. 5,212,091. Analogs of TFPI having portions of TFPI and TFPI -2, fragments of TFPI comprising the first and second Kunitz-type domains, as well as fragments of TFPI comprising the first and second Kunitz domains and a heparin binding region may all be useful in the method of the invention. Such analogs and fragments are described in U.S. 5,106,833 as well as U.S.S.N. 08/286,521. One such fragment is TFPI(1-160) having the first 160 amino acids of mature TFPI.

As used herein, "pharmaceutically acceptable composition" refers to a composition that does not negate or reduce the biological activity of formulated TFPI, and that does not have any adverse biological effects when formulated TFPI is administered to a patient.

As used herein, "patient" encompasses human and veterinary patients.

B. General Methods

TFPI may be prepared by recombinant methods as disclosed in U.S. 5,212,091, the disclosure of which is herein incorporated by reference. Briefly, TFPI is expressed in

Escherichia coli cells and the inclusion bodies containing TFPI are isolated from the rest of the cellular material. The inclusion bodies are subjected to sulfitolysis, purified using ion exchange chromatography, refolded by disulfide interchange reaction and the refolded, active TFPI purified by cation exchange chromatography. TFPI may also be produced in yeast as disclosed in co-pending U.S.S.N. 08/286,530.

<u>Whole Blood Culture</u>

The whole blood culture system can be carried out as follows. Blood is collected from normal donors into anticoagulant. Venous blood from normal health donors was collected directly into clinical heparin or EDTA (K3) vacutainers (Baxter). Alternatively, venous blood was collected into sterile polypropylene syringes and immediately transferred into microtiter wells containing indicated concentrations of various additives including:

a. 20-50U/ml heparin (blood fully anticoagulated, even with 10X dilution)

b. 50-60 U/ml hirudin (recombinant yeast, American Diagnostica)

c. 10 μg/ml TFPI

d. 1 U/ml heparin (ESI)

e. 10 mM EDTA (for isolated neutrophils)

f. 1 ng/ml LPS (*E. coli* Rc) (Sigma, St. Louis, MO)

g. 3.8% citrate (for isolated PBMC).

TFPI was formulated at 11mg/ml in 2M urea, 20mM sodium phosphate pH 7.2 and 0.14M NaCl. Blood collected into vacutainers was quickly transferred into polypropylene tubes prior to addition into culture wells.

Whole blood was cultured in 96 well microtiter plates (Corning) at a volume of 200 µl per well at 37C, 5% CO_2 for 2-48 hours in a humidified atmosphere. The blood will typically be at a final dilution of 1:8 to 1:10 in RPMI 1640 medium + 0.1% "low-endotoxin" fetal calf serum (FCS) (Hyclone, Logan, UT). The cultures were then spun down at 400 x g for 1 minute at 4°C. Supernatant liquids were then removed at various time points (typically 2-2.5 hours) for analysis of soluble mediators. In the event that clotting occurred during culturing, the contents of clotted wells and comparative groups were transferred to polypropylene microfuge tubes and briefly spun to pellet cells and fibrin clot prior to harvesting supernatants. Soluble mediators were measured in supernatants by ELISA or other bioassay.

Peripheral Blood Mononuclear Cell (PBMC) Cultures

Whole blood was collected into EDTA vacutainers was layered over a ficoll gradient (NIM medium, Cardinal Assoc.) at a mixture of 7-8 ml blood onto 5 ml NIM medium in 15 ml polystyrene tubes. The tubes were spun at 500 x g for 30 minutes and the mononuclear cell layer was isolated as the top band in the gradient. In some experiments, PBMC were also isolated using citrated Cell Preparation Tubes (Becton-Dickinson, Mountain View, CA) wherein blood is collected and fractionated in the same tube. Identical results were obtain utilizing PBMC isolated in either manner. Following a sterile saline wash, PBMC were cultured at ~1 x 10^5 cells per well in RPMI/0.1% FCS as described above for whole blood cell cultures.

Assay for Soluble Mediators

ELISA assays for elastase, IL-8, IL-6 and TNF were conducted as follows. 96 well microtiter plates were coated overnight with the appropriate antibodies. Plates were

washed and samples were added to each well along with biotin-labelled antibody and serum. The plates were then incubated and washed. Streptavidin-horseradish peroxidase was then added to the wells and allowed to incubate. The wells were again washed and developed with TMB, sodium acetate and peroxide. The reaction was stopped by the addition of 2M sulfuric acid and plates read at O.D. 450 nm. When assaying for elastase, there is an incubation period between the addition of the samples and the biotinylated anti-elastase antibodies. For TNF, poly-streptavidin-horseradish peroxidase and milk are used instead of Strep-HRP and serum.

Spectrozyme Plasmin assay kits were obtained from American Diagnostica. Quantikine IL-1β ELISA kits were purchased from R&D Systems. The manufacturers' instructions were followed in completing the assays.

Coagulation Activation

Measurement of the extent of coagulation activation was performed in a qualitative manner by observation of clotting and, quantitatively, via immunodetection of thrombin:antithrombin (TAT) complex and fibrinopeptide A levels according to manufacturer's protocols (Diagnostica Stago, France). Supernatants were also routinely analyzed for chromogenic activity against various substrates including Spectrozyme Xa and TH (thrombin) (American Diagnostica) for correlation with the above metrics as well as to confirm activity of purified factors added to isolated PBMC cultures including prothrombin, α-thrombin, and factor Xa.

C. Examples

Example 1

A culture system utilizing normal human blood wherein coagulation activation and clotting could be controlled and inflammatory mediator responses could be evaluated in the presence or absence of LPS was established. Essentially, blood is collected in concentrations of anticoagulant, such as the irreversible thrombin inhibitor hirudin. When cultured at final blood dilutions of 1:10, coagulation activation and clotting could be observed. The extent of coagulation activation and clotting can be controlled by appropriate additions of anticoagulants upon blood dilution.

While most examinations of the effect of coagulation activation/clotting on elastase release have been performed in 1:10 diluted blood, the phenomena was observed in undiluted whole blood as indicated in Figure 1. The incubation was carried out for two hours. Clotting the whole blood resulted in significant elastase release in the supernatant as compared to blood treated with 50 U/ml heparin. In undiluted whole blood, the addition of LPS resulted in only a small increment in elastase production, probably because the coagulation signal itself is so strong in the culture. TFPI addition at t=0 abrogates the coagulation and coagulation+LPS induced elastase release.

As shown in Figure 2, dilution of hirudin such that coagulation activation/clotting through thrombin can proceed is accompanied by elastase release which is most marked at low hirudin concentrations (5 & 10 U/ml) wherein clotting can be observed the time of harvest (t=2 hours). The addition of low concentrations of TFPI to hirudin-treated blood cultures (5 U/ml hirudin) blocks elastase release in a dose-dependent manner (Figure 3).

Addition of LPS in the low-hirudin culture results in significantly more elastase production (Figure 4). Nonetheless, TFPI markedly inhibits elastase release.

In contrast to anticoagulated blood collected in heparin, blood collected into TFPI fails to exhibit the synergistic elastase release observed upon culture with LPS. Moreover, the coagulation/LPS-induced elastase release in heparin-anticoagulated blood can be inhibited by the addition of TFPI to the culture at t=0 (Figure 4).

Finally, the effect of the presence of TFPI in the cultures on synthesis and release of plasmin was determined. Figure 5 shows that increasing concentrations of TFPI result in decreased detection of plasmin activity in the cultures. The inhibitory effect of TFPI on plasmin activity may therefore serve as a marker for efficacy of TFPI in patients.

Example 2

Using the culture system described above, it has been found that IL-8 production increases as a result of the coagulation activation/clotting. Also, coagulation activation/clotting and LPS appear to have a synergistic effect for IL-8 synthesis and release.

Dilution of hirudin in the blood cultures allows thrombin amplification of the coagulation cascade resulting in significant coagulation activation and observable clotting. Coincident with coagulation activation/clotting is the production of IL-8 into culture supernatants detectable by ELISA (Figure 6). TFPI inhibits the coagulation activation/clotting-induce IL-8 production in a dose dependent manner (Figure 6).

When LPS (1 ng/ml) is included in the hirudin-treated blood cultures, a synergistic increase in IL-8 production is observed under conditions where significant coagulation activation/clotting occurs (i.e. 5 - 10 U/ml hirudin) (Figure 7). The response is synergistic

because coagulation activation/clotting results in ~350pg/ml IL-8 and LPS induces ~450 pg/ml IL-8 under fully anticoagulated conditions (50 U/ml hirudin or 5 U/ml heparin), but the combinations of coagulation activation/clotting and LPS results in ~2500 pg/ml IL-8 production. As shown in Figure 7, TFPI inhibits the synergistic IL-8 production in a dose-dependent manner.

The ability of TFPI to abrogate IL-8 production induced by coagulation activation/clotting or the combination of coagulation activation/clotting + LPS is not due to altered kinetics of cytokine production (Figures 8 and 9). TFPI inhibits induced IL-8 production at all time points evaluated.

The described IL-8 response to the combination of coagulation activation/clotting + LPS is somewhat unique as the combination does not result in synergistic production of TNFα, IL-6 or IL-1β (Figure 10). Moreover, production of TNFα or IL-1β induced in low-hirudin+LPS cultures is not significantly inhibited by added TFPI concentrations to 10 μg/ml. While IL-6 production is slightly inhibited by TFPI, the IL-8 response is most significantly reduced by TFPI. The mechanism for the TFPI effect on induced IL-8 production in these cultures has not been determined. Without being bound to any particular theory, it may be that the ability of TFPI to inhibit coagulation activation by direct inhibition of factor Xa and the inhibition of factor VIIa/tissue factor in a Xa-dependent manner. However, it may be that TFPI has some ability to directly inhibit LPS activity.

Example 3

Adult Respiratory Distress Syndrome (ARDS) is an acute inflammatory process characterized by neutrophil accumulation and edema in the lungs, as well as progressive

hypoxemia. Repine, (1992) Lancet 339:466-469. ARDS is an inflammatory disease occurring as a complicating factor in a number of diseases including sepsis. Patients diagnosed with ARDS can be treated by administration of an effective amount of TFPI. The dosage of TFPI will vary according to a number of factors including progress of ARDS (soon after onset to late stage disease), patient size and other factors known to and appreciated by those skilled in the art.

Patients at risk for developing ARDS may be identified by chest X-ray. Opaqueness of the radiogram lung area is indicative of neutrophil migration into the lung and is an accepted clinical diagnostic hallmark of ARDS.

Example 4

The inhibitory effect of TFPI on the synthesis and release of neutrophil elastase, IL-8 and on plasmin may be used to assess the efficacy of treatment in patients with thrombosis disorders, patients with diseases associated with increased neutrophil elastase and in patients with diseases associated with increased IL-8. It is believed that the levels of neutrophil elastase, IL-8 and plasmin may be predictive of patient responsiveness to TFPI and prognosis. Patients who have received TFPI will have blood drawn and assayed for neutrophil elastase levels, for IL-8 levels, for plasmin activity or for any combination of these indicators. Levels for each of these indicators may be compared to an established historical baseline, as determined by sampling of populations of normal human volunteers. Alternatively, the level of neutrophil elastase, IL-8 or plasmin per patient may be followed over time prior to and after administration of TFPI. In the event that levels of the indicator or indicators tested have not decreased, additional dosing with TFPI may be required.

Claims

We claim:

1. A method of treating disease associated with increased synthesis and release of neutrophil elastase, comprising administering an agent, said agent capable of inhibiting coagulation and further capable of inhibiting release of neutrophil elastase, to a patient displaying the symptoms of said disease associated with increased expression of neutrophil elastase.

2. The method of claim 1 wherein the disease is an inflammatory disease.

3. The method of claim 2 wherein the disease is selected from the group consisting of:

   (a) severe acute pancreatitis;

   (b) emphysema;

   (c) rheumatoid arthritis;

   (d) multiple organ failure;

   (e) cystic fibrosis;

   (f) sepsis; and

   (g) ARDS.

4. The method of claim 3 wherein the disease is ARDS.

5. The method of claim 1 wherein the agent is TFPI.

6. The method of claim 1 wherein the agent is a mutein of TFPI.

7. The method of claim 1 wherein the agent is ala-TFPI.

8. The method of claim 1 wherein the agent is a fragment of TFPI capable of inhibiting coagulation and further capable of inhibiting release of neutrophil elastase.

9. A method of preventing disease associated with increased synthesis and release of neutrophil elastase, comprising administering an agent, said agent capable of inhibiting coagulation and further capable of inhibiting release of neutrophil elastase, to a patient at risk for developing said disease associated with increased expression of neutrophil elastase.

10. The method of claim 9 wherein the disease is an inflammatory disease.

11. The method of claim 10 wherein the disease is selected from the group consisting of:

(a) severe acute pancreatitis;

(b) emphysema;

(c) rheumatoid arthritis;

(d) multiple organ failure;

(e) cystic fibrosis;

(f) sepsis; and

(g) ARDS.

12. The method of claim 11 wherein the disease is ARDS.

13. The method of claim 9 wherein the agent is TFPI.

14. The method of claim 9 wherein the agent is a mutein of TFPI.

15. The method of claim 9 wherein the agent is ala-TFPI.

16. The method of claim 9 wherein the agent is a fragment of TFPI capable of inhibiting coagulation and further capable of inhibiting release of neutrophil elastase.

17. A method of treating disease associated with increased synthesis and release of IL-8, comprising administering an agent, said agent capable of inhibiting coagulation and

further capable of inhibiting release of IL-8, to a patient displaying the symptoms of said disease associated with increased expression of IL-8.

18. The method of claim 17 wherein the disease is an inflammatory disease.

19. The method of claim 17 wherein the disease is selected from the group consisting of:

(a) ARDS;

(b) reperfusion injury;

(c) sepsis; and

(d) arthritis.

20. The method of claim 19 wherein the disease is ARDS.

21. The method of claim 17 wherein the agent is TFPI.

22. The method of claim 17 wherein the agent is a mutein of TFPI.

23. The method of claim 17 wherein the agent is ala-TFPI.

24. The method of claim 17 wherein the agent is a fragment of TFPI capable of inhibiting coagulation and further capable of inhibiting release of IL-8.

25. A method of preventing disease associated with increased synthesis and release of IL-8, comprising administering an agent, said agent capable of inhibiting coagulation and further capable of inhibiting release of IL-8, to a patient at risk for developing said disease associated with increased expression of IL-8.

26. The method of claim 25 wherein the disease is an inflammatory disease.

27. The method of claim 26 wherein the disease is selected from the group consisting of:

(a) ARDS;

(b) reperfusion injury;

(c) sepsis; and

(d) arthritis.

28. The method of claim 27 wherein the disease is ARDS.

29. The method of claim 25 wherein the agent is TFPI.

30. The method of claim 26 wherein the agent is a mutein of TFPI.

31. The method of claim 27 wherein the agent is ala-TFPI.

32. The method of claim 28 wherein the agent is a fragment of TFPI capable of inhibiting coagulation and further capable of inhibiting release of IL-8.

33. A method for measuring patient responsiveness to and efficacy of TFPI, comprising:

(a) determining the level or activity of one or more indicators of TFPI efficacy, said one or more indicators selected from the group consisting of neutrophil elastase; IL-8; and plasmin.

34. The method of claim 33 wherein said one or more indicators are neutrophil elastase; IL-8; and plasmin.

35. The method of claim 33 wherein the patient has been diagnosed with one or more diseases associated with increased synthesis and release of neutrophil elastase:

(a) severe acute pancreatitis;

(b) emphysema;

(c) rheumatoid arthritis;

(d) multiple organ failure;

(e) cystic fibrosis;

(f) sepsis; and

(g) ARDS.

36. The method of claim 33 wherein the patient has been diagnosed with one or more diseases associated with increased synthesis and release of IL-8:

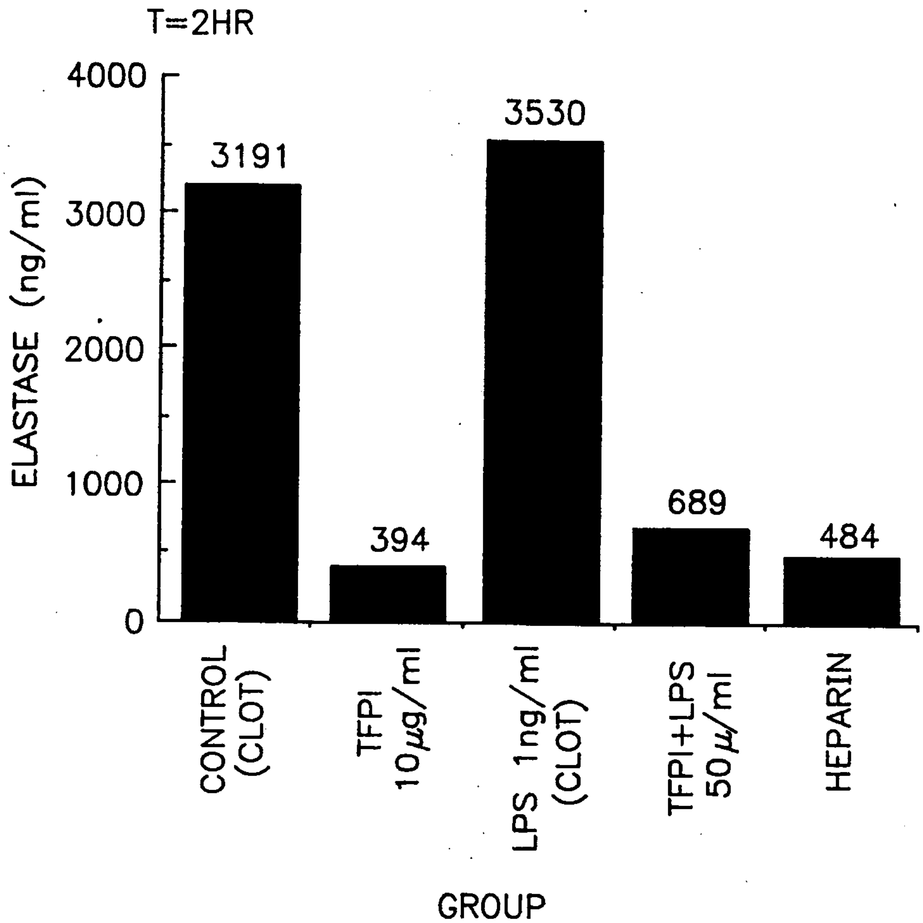
(a) ARDS;

(b) reperfusion injury;

(c) sepsis; and

(d) arthritis.

37. The method of claim 33 wherein the patient has been diagnosed with a thrombosis related syndrome.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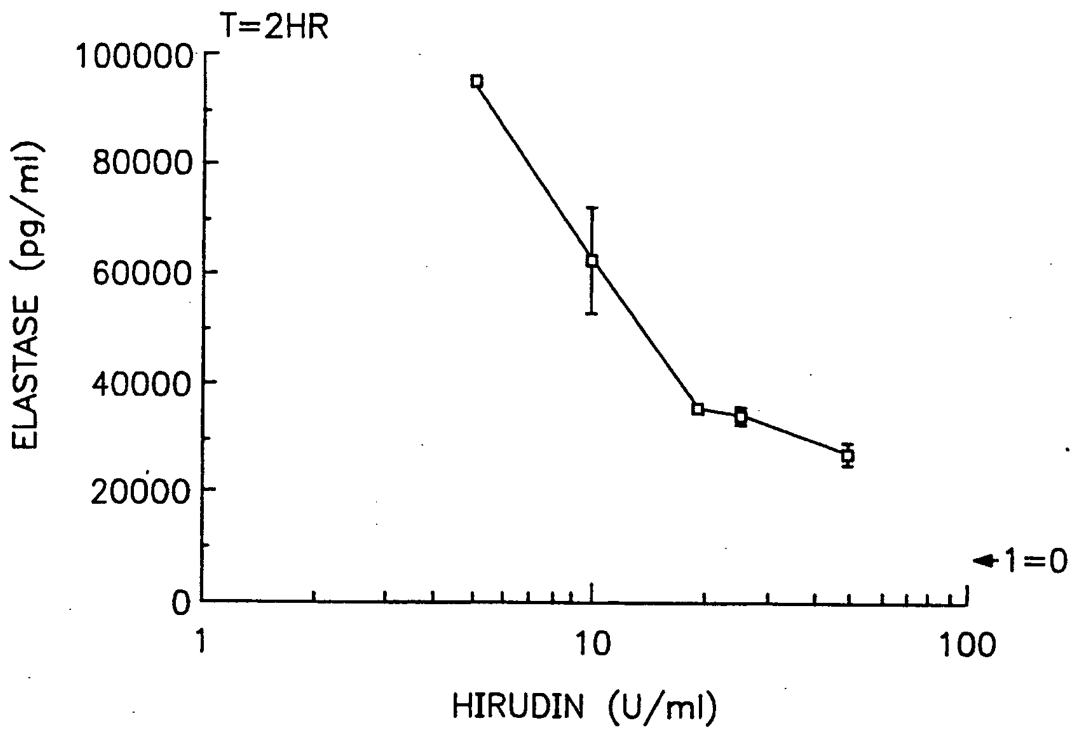
TFPI+LPS 50μ/ml
HEPARIN
GROUP

FIG. 1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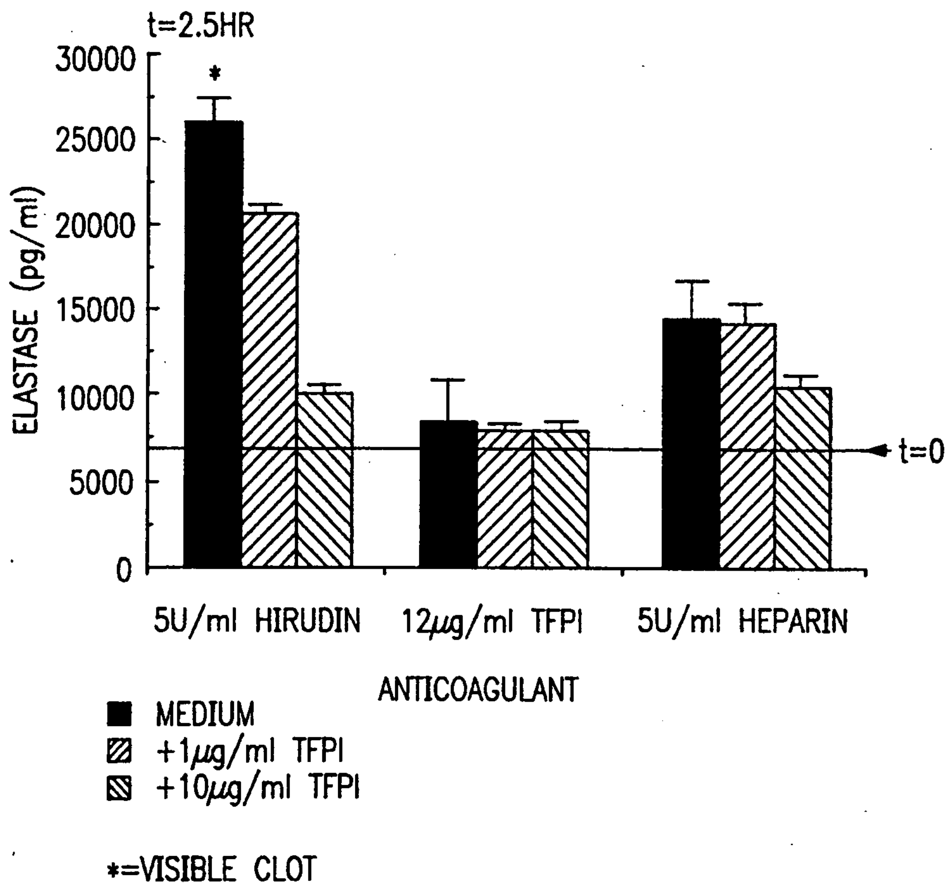
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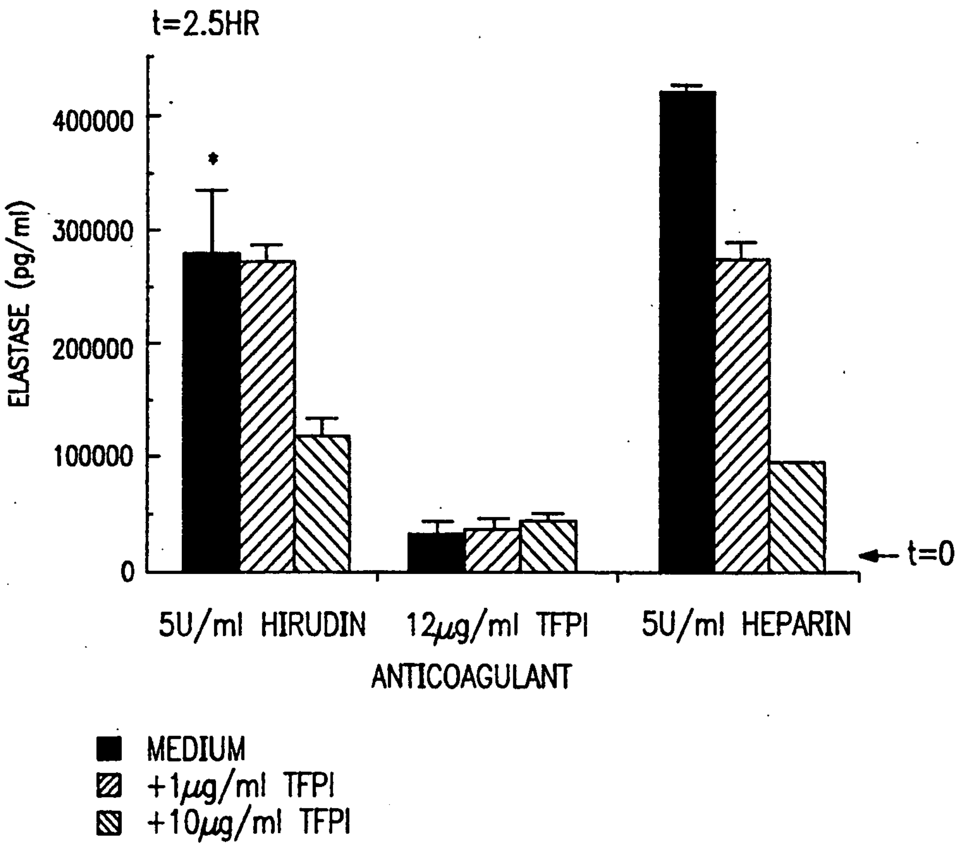
FIG. 3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5U/ml HEPARIN
ANTICOAGULANT
MEDIUM
+1μg/ml TFPI
+10μg/ml TFPI

FIG. 4

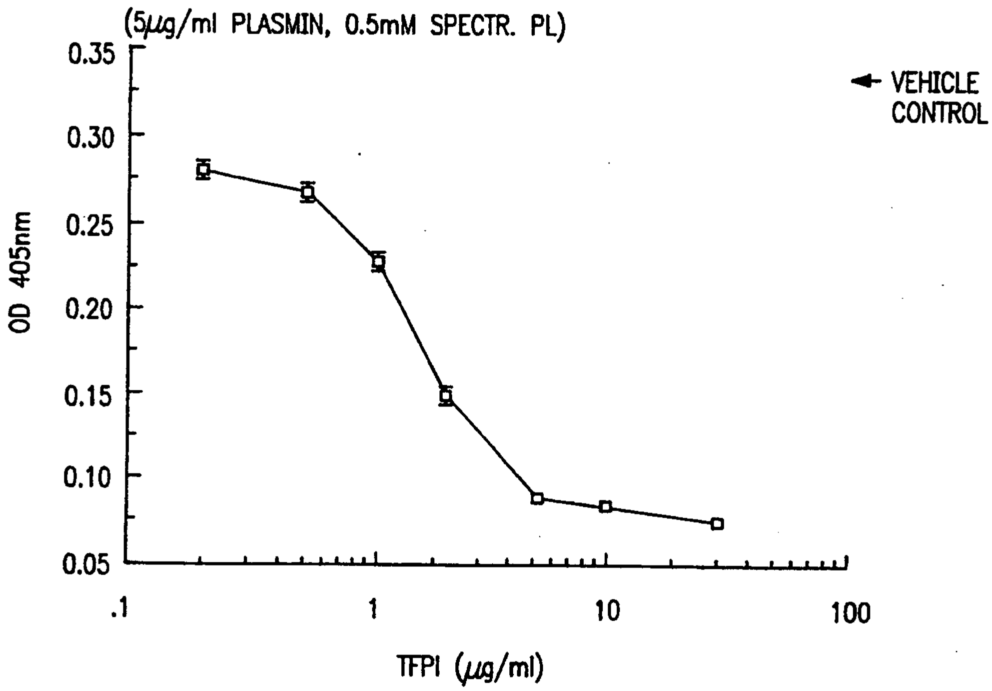
(5μg/ml PLASMIN, 0.5mM SPECTR. PL)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
OD 405nm
← VEHICLE CONTROL
.1
1
10
100
TFPI (μg/ml)

FIG. 5

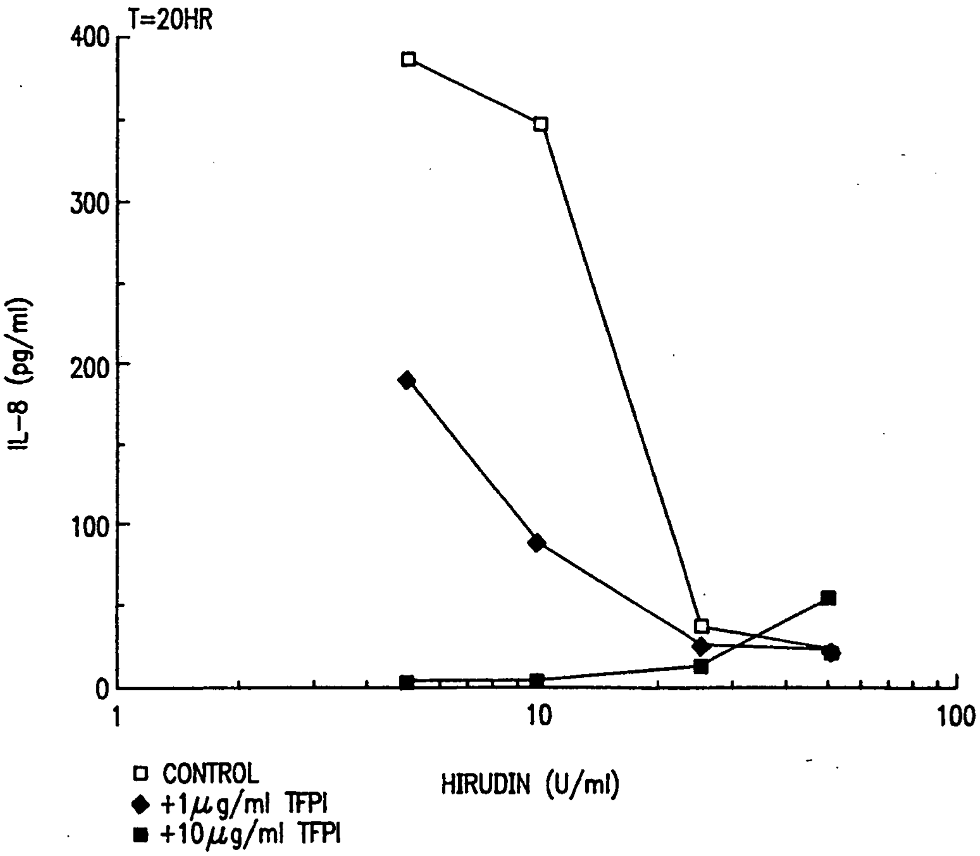
T=20HR
400
300
200
100
0
IL-8 (pg/ml)
1
10
100
HIRUDIN (U/ml)
CONTROL
+1μg/ml TFPI
+10μg/ml TFPI

FIG. 6

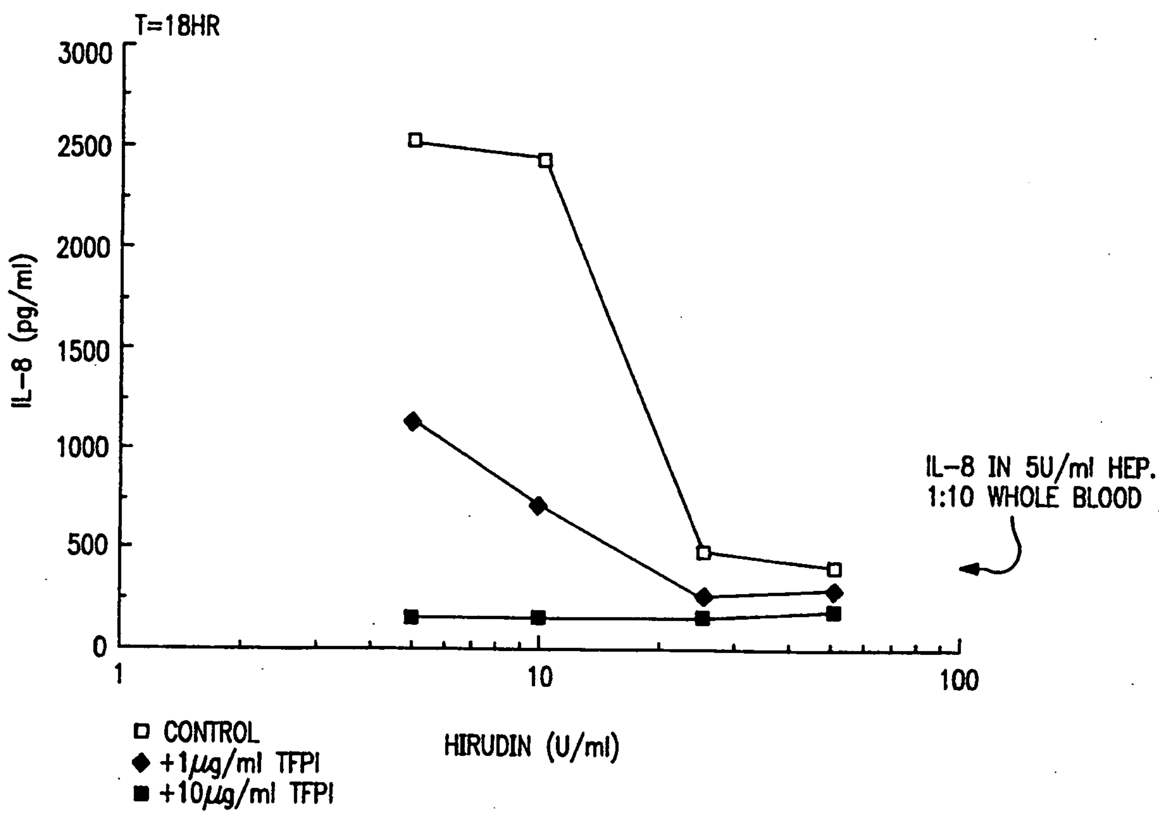
T=18HR
3000
2500
2000
1500
1000
500
0
IL-8 (pg/ml)
1
10
100
HIRUDIN (U/ml)
IL-8 IN 5U/ml HEP.
1:10 WHOLE BLOOD
CONTROL
+1μg/ml TFPI
+10μg/ml TFPI

FIG. 7

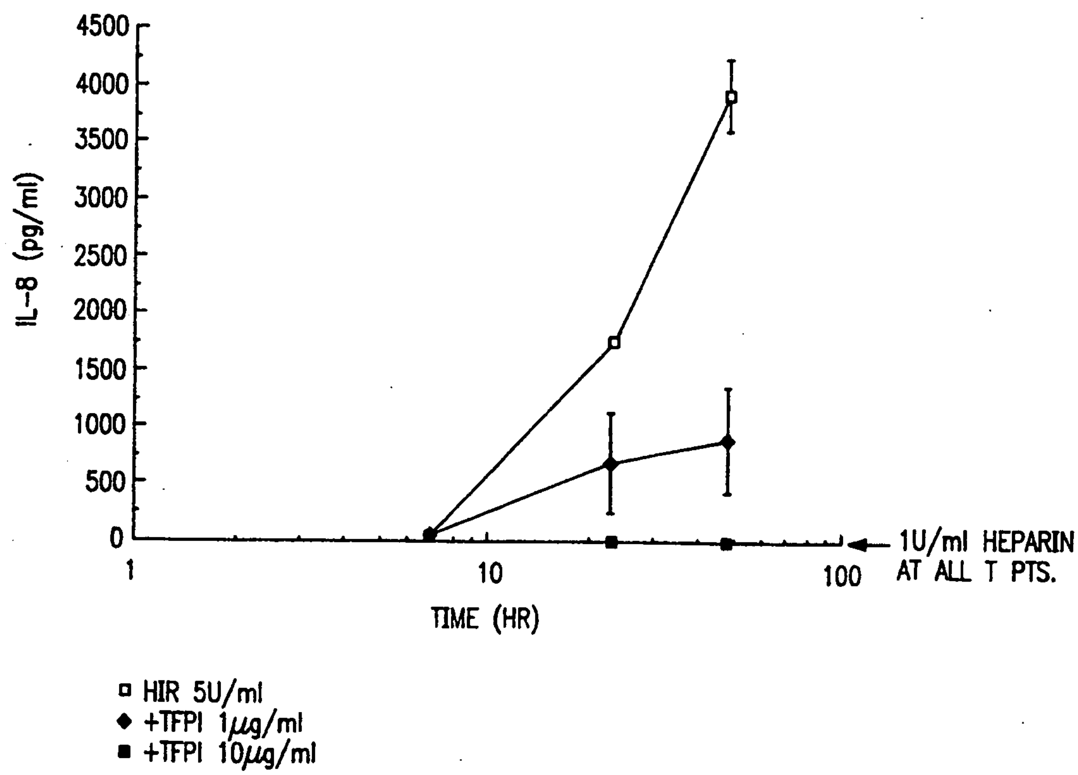
4500
4000
3500
3000
2500
2000
1500
1000
500
0
IL-8 (pg/ml)
1
10
100
TIME (HR)
1U/ml HEPARIN
AT ALL T PTS.
HIR 5U/ml
+TFPI 1μg/ml
+TFPI 10μg/ml

FIG. 8

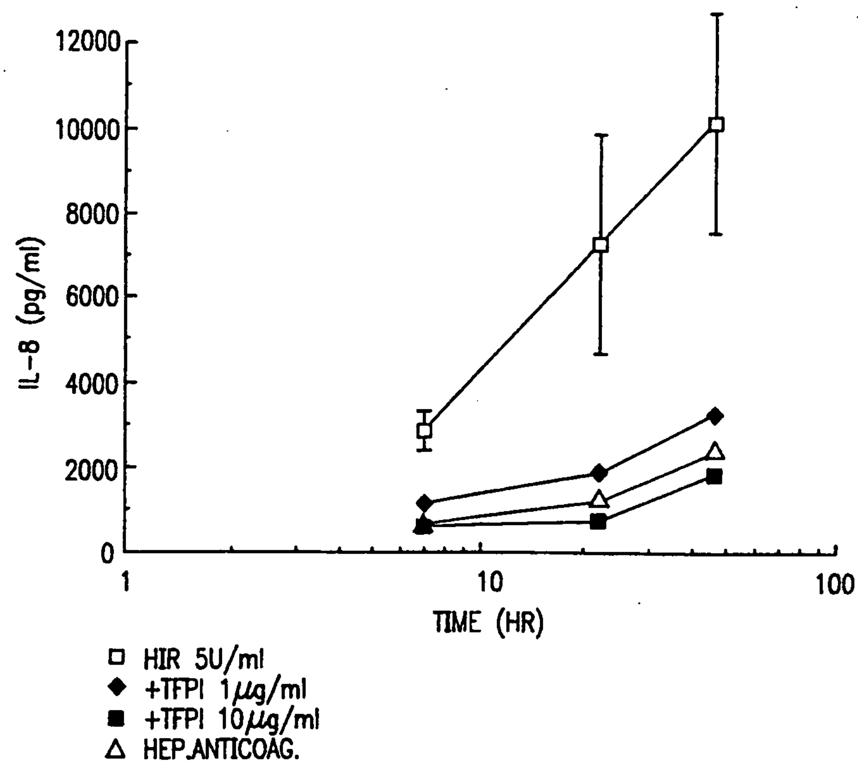
12000
10000
8000
6000
4000
2000
0
IL-8 (pg/ml)
1
10
100
TIME (HR)
HIR 5U/ml
+TFPI 1μg/ml
+TFPI 10μg/ml
HEP.ANTICOAG.

FIG. 9

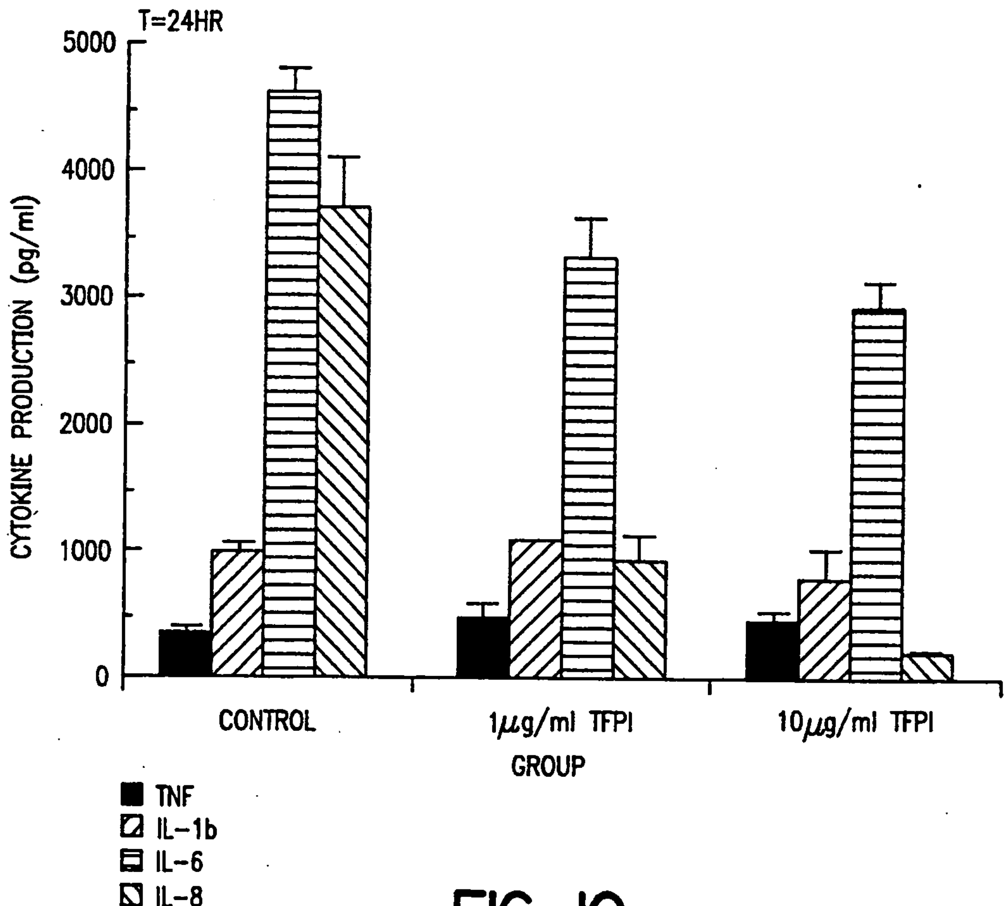
T=24HR
5000
4000
3000
2000
1000
0
CYTOKINE PRODUCTION (pg/ml)
CONTROL
1μg/ml TFPI
10μg/ml TFPI
GROUP
TNF
IL-1b
IL-6
IL-8

FIG. 10

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/57

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 24143 (CETUS ONCOLOGY CORPORATION) 9 December 1993 see the whole document ---	1-32
X	WO,A,93 14122 (NOVO NORDISK A/S) 22 July 1993 see the whole document ---	1-32
X	CIRCULATORY SHOCK 44 (3). 1994. 126-137, XP000602149 CARR C ET AL: "Recombinant E. coli-derived tissue factor pathway inhibitor reduces coagulopathic and lethal effects in the baboon gram-negative model of septic shock." see the whole document ---	1-32

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
19 September 1996	27.09.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THROMB HAEMOSTASIS 67 (5). 1992. 537-541, XP000602163 PETERSEN L C ET AL: "EFFECT OF LEUKOCYTE PROTEINASES ON TISSUE FACTOR PATHWAY INHIBITOR." see the whole document ---	1-32
P,X	WO,A,95 18830 (PROTEIN ENGINEERING CORPORATION) 13 July 1995 see the whole document ---	1-32
P,X	WO,A,96 06637 (WASHINGTON UNIVERSITY) 7 March 1996 see the whole document ---	1-32
P,X	37TH ANNUAL MEETING OF THE AMERICAN SOCIETY OF HEMATOLOGY, SEATTLE, WASHINGTON, USA, DECEMBER 1-5, 1995. BLOOD 86 (10 SUPPL. 1). 1995. 875A, XP002013723 JOHNSON K ET AL: "The cytokine response to coagulation and endotoxin in whole blood: Regulation by the tissue factor pathway inhibitor." see the whole document -----	1-37

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Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1 - 32 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9324143	09-12-93	CA-A-	2136953	09-12-93
		EP-A-	0643585	22-03-95
		JP-T-	7507300	10-08-95
WO-A-9314122	22-07-93	AU-A-	3346093	03-08-93
		CA-A-	2127246	22-07-93
		CZ-A-	9401644	15-12-94
		EP-A-	0621872	02-11-94
		FI-A-	943234	06-07-94
		HU-A-	70293	28-09-95
		JP-T-	7504891	01-06-95
		NO-A-	942549	07-09-94
		ZA-A-	9300096	10-08-93
WO-A-9518830	13-07-95	NONE		
WO-A-9606637	07-03-96	AU-A-	3410195	22-03-96